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(54) Title: DOMINANT NEGATIVE MUTANTS OF IRS-1 AND USES THEREOF (57) Abstract Dominant negative mutants of mammalian IRS-1 proteins and therapeutic compositions containing such mutants. Also featured are methods of using the dominant negative mutants to inhibit tyrosyl phosphorylation of endogenous IRS-1 in mammalian cells and methods of treating a mammalian malignancy in which tyrosyl phosphorylation of endogenous IRS-1 plays a causative role.		

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DOMINANT NEGATIVE MUTANTS OF IRS-1 AND USES THEREOF

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5 35711 and AA-02666 from the National Institutes of
Health. The government may have certain rights in the
invention.

Field of the Invention

This invention relates to signal transduction in
10 mammalian cells.

Background of the Invention

Human hepatocellular carcinoma (HCC) is one of the
most common and least understood tumors. Although
persistent hepatitis B and C virus infections are major
15 risk factors for the development of this disease, little
is known regarding cellular pathogenesis. Normal
hepatocyte proliferation is regulated by several growth
factors, of which insulin, epidermal growth factor (EGF),
transforming growth factor α (TGF α) and hepatocyte growth
20 factor (HGF) are believed to be the most important
(Moradpour et al., IN: *Hepatology*, 3rd ed., W. B.
Saunders, Philadelphia, PA). Such growth factors bind to
hepatocyte cell surface receptors with intrinsic tyrosine
kinase activity and initiate a series of protein
25 phosphorylation events within the cells. Tyrosyl
phosphorylation (TP) of downstream molecules transmits
the mitogenic signals from the cell surface to the
nucleus through several signal transduction pathways.

A cDNA encoding one of the key molecules involved
30 in the insulin mediated signal transduction cascade,
human insulin receptor substrate-1 (human IRS-1 or hIRS-
1; SEQ ID NO:1), has been found to be overexpressed at
the protein and RNA levels in HCC cell lines and tumor

tissues (Furusaka et al., *Mol. Cell Biol.* 11:4405-4414, 1991; Nishiyama et al., *Biochem. Biophys. Res. Commun.* 183:280-285, 1992). Tyrosine residues of IRS-1 are phosphorylated following cellular stimulation by ligands
5 such as insulin; insulin-like-growth-factor 1 (IGF-1); interleukins 4, 9 and 13; interferons α and β ; growth hormone; leukemia inhibitory factor; and tumor necrosis factor (Artersinger et al., *J. Biol. Chem.* 270:14685-14692, 1995; Guo et al., *J. Biol. Chem.* 271:615-618,
10 1996; Myers et al., *J. Biol. Chem.* 270:11715-11718, 1995; Plataniias et al., *J. Biol. Chem.* 271:278-282, 1996; Welham et al., *J. Biol. Chem.* 270:12286-12296, 1995). Tyrosyl phosphorylated IRS-1 serves as a key "docking" protein. It transmits mitogenic or metabolic signals by
15 interacting, through specific motifs, with downstream molecules containing the Src homology domain 2 (SH₂) (Sun et al., *Nature* 377:173-177, 1995). For example, the ⁸⁹⁷YVNI (SEQ ID NO:2) motif of hIRS-1 binds to the Grb2 adapter protein (Baltensperger et al., *Science* 260:1950-
20 1952, 1993); the ¹¹⁸⁰YIDL (SEQ ID NO:3) motif binds to Syp phosphatase (also known as PTP1D, PTP2C, and SH-PTP2) (Kuhne et al., *J. Biol. Chem.* 268:11479-11481, 1993); and ⁶¹³YMPM (SEQ ID NO:4) and ⁹⁴²YMKM (SEQ ID NO:5) motifs are the principal binding sites for the p85 subunit of
25 phosphatidylinositol-3 kinase (PI3K) (Backer et al., *EMBO J.* 11:3469-3479, 1992; Myers et al., *Proc. Natl. Acad. Sci. USA* 89:10350-10354, 1992). While TP sites are recognized throughout the entire IRS-1 protein, the SH₂-binding domains are located only in the C-terminal
30 region (Myers et al., *Trends Biochem. Sci.* 19:289-293, 1994). The N-terminal sequences, however, contain three important functional domains identified as a pleckstrin homology (PH) region, located at amino acid residues 9-117 (Musacchio et al., *Trends Biochem. Sci.* 18:343-348,
35 1993), and two regions homologous to a phosphotyrosine

binding (PTB) domain, located at amino acid residues 161-317 (Sun et al., *Nature* 377:173-177, 1995) and at amino acid residues 314-463 (Gustafson et al., *Mol. Cell. Biol.* 15:2500-2508, 1995).

5 Normal hepatic growth has been associated with TP of IRS-1 and its subsequent interaction with SH₂-containing molecules such as Grb2 and PI3K during the G1 phase of the hepatocyte cell cycle following partial hepatectomy (Sasaki et al., *J. Biol. Chem.* 268:3805-3808,
10 1993). There is now evidence to support the hypothesis that IRS-1 may have transforming properties as well (D'Ambrosio et al., *Cell. Growth Differ.* 6:557-562, 1995; Ito et al., *Mol. Cell. Biol.* 16:943-951, 1996). Stable transfection and overexpression of the hIRS-1 gene in NIH
15 3T3 cells leads to increased TP of the protein, enhanced binding of the protein to Grb2 and Syp but not PI3K, and persistent activation of the downstream MAPK (mitogen-activated protein kinase) cascade. Such transfected cells develop a phenotype characterized by increased
20 transformed foci formation, induction of anchorage independent cell growth, increased cell proliferation and formation of large tumors in nude mice (Ito et al., *Mol. Cell Biol.* 16:943-951, 1996). The functional domains of the hIRS-1 protein required for its transforming activity
25 have been shown to reside in both the ⁸⁹⁷YVNI (SEQ ID NO:2) and ¹¹⁸⁰YIDL (SEQ ID NO:3) motifs (Tanaka et al., *J. Biol. Chem.*, 1996). Insulin and IGF-1 have been shown to act as dominant cellular mitogens for several different human tumors including HCC (Macaulay, *Br. J. Cancer*
30 65:311-320, 1992).

Summary of the Invention

The present invention is based on the discovery of the functional domains of mammalian IRS-1's that are essential for insulin- and IGF-1-induced TP and for

subsequent activation of downstream signal transduction molecules associated with tumorigenicity. Applicants have also discovered that a dominant negative mutant protein derived from hIRS-1 blocks TP of endogenous
5 hIRS-1 protein and other substrates of insulin- or IGF-1-induced TP (e.g., Shc). This mutant protein also reverses the malignant phenotype of HCC cells.

Accordingly, the invention features dominant negative mutants of mammalian (e.g., human) IRS-1
10 proteins. The mutants, when co-expressed with a wild type IRS-1 in a cell, block the function of the wild type IRS-1 in the cell. Dominant negative mutants substitute for wild-type proteins implicated in pathogenicity and can counteract their pathogenic effects.

15 The mutants of the invention can contain the pleckstrin homology domain and the two phosphotyrosine binding domains of their corresponding wild type IRS-1's. For instance, a dominant negative mutant of human IRS-1 can contain 460 amino acid residues from the amino-
20 terminal half (i.e., amino acid residues 1-621) of native IRS-1. The pleckstrin homology (PH) domain corresponds approximately to amino acid residues 9-117 of hIRS-1, and is believed to bring IRS-1 to close proximity to cell membranes on which insulin receptor resides. The two
25 phosphotyrosine domains (PTB) correspond approximately to amino acid residues 161-314 and 315-463 of hIRS-1, respectively. The PTB domains, when bound to insulin receptor, become phosphorylated at their tyrosine residues. IRS-1 thus phosphorylated can transmit the
30 signal from the insulin receptor via the IRS-1' SH₂-binding region to proteins residing downstream in the insulin/IGF-1 signal transduction pathway.

The mutant protein may also lack at least one or even all of the functional SH₂-binding motifs at its SH₂-
35 binding region, so that the protein can no longer bind to

the SH₂ domain of one or all of its adaptor proteins (e.g., Syp, Grb2, PI3K, or NCK). For instance, a dominant negative mutant of human IRS-1 may lack at least 300 amino acid residues from its carboxy-terminal half (i.e., amino acid residues 622-1243). It may even lack the last 727 amino acid residues of human IRS-1.

The mutant can additionally contain a heterologous sequence, i.e., a sequence not related to IRS-1, to facilitate its identification or purification. The heterologous sequence may contain an epitope to which an antibody can bind, or a ligand (e.g., a maltose-binding protein domain or a His tag) of any other receptor molecule (e.g., maltose or nickel). This sequence can range from, e.g., 4-25 amino acid residues in length, and replace 0-25 amino acid residues of the IRS-1 sequence in the mutant protein. It will typically be at one end of the mutant protein, but can be in the middle. Exemplary epitopes include FLAG (SEQ ID NO:6), E-tag, c-myc tag, VSV-GP, T7 tag, HSV tag, and HA tag, all of which are well known in the art. The identity of the tag sequence is not critical.

Due to polymorphism that may exist at the IRS-1 genetic locus, minor variations in the amino acid sequence of the IRS-1's found in any given mammalian species may occur. For purposes of this invention, as long as adequate dominant negative effect on wild type IRS-1 remains, mutant proteins containing minor amino acid sequence variations as a result of natural IRS-1 polymorphism, or even as a result of recombinant genetic manipulation, are within the scope of this invention. In particular, one or all of the region corresponding to residues 118-160 of hIRS-1 (i.e., between the PH domain and the closest PTB domain) can be deleted or substituted with alternative residues. Preferably 0-35 of those

residues would be deleted or substituted, and more preferably 0-25 (e.g., 0-10 or 0-5).

This invention also includes methods of inhibiting tyrosyl phosphorylation of an IRS-1 in a mammalian cell (e.g., a human hepatic cell). In these methods, an effective amount of a mutant of the invention is introduced into the cell (e.g., by expression from a recombinant expression construct within the cell). An effective amount is an amount that decreases the insulin- or IGF-1-induced TP of the endogenous IRS-1.

The dominant negative mutants of the invention can be used to reverse (e.g., eliminate or mitigate) a malignant phenotype of mammalian tumors (e.g., brain cancer, lung cancer, pancreatic cancer, and gastrointestinal cancers such as colon, liver cancer, or stomach cancer) in which hyperactivity of IRS-1 is known or believed to play a causative role. The malignant phenotype can be characterized by, e.g., growth without contact inhibition, anchorage-independent growth, increased rate of proliferation as compared to a normal cell, or ability to form a tumor in a nude mouse.

Also within the scope of the invention are therapeutic compositions containing a polypeptide of the invention, or a DNA encoding the polypeptide, admixed with a pharmaceutically acceptable carrier.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of

conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

- 5 Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawing

- Fig. 1 is a schematic diagram showing the functional domains of the hIRS-1 protein and structures of the truncated mutants. The various C-terminal deletion mutants (i.e., the ΔC-hIRS-1 series) each contain a FLAG (F) epitope at the C-terminus. "PH" and "PTB" indicate pleckstrin homology and phosphotyrosine binding regions, respectively. "ΔN" and "ΔC" denote N- and C-terminal truncated mutants, respectively. The amino acid residues deleted in each mutant are indicated after the sign "Δ" in the name of the mutant.
- 10
15

Detailed Description of the Invention

- Described in the Example below is one exemplary inhibitory protein of the invention. This protein, i.e., hIRS-1Δ517-1243, is a dominant negative mutant of hIRS-1, and consists of the 516 extreme N-terminal residues of wild type hIRS-1 and a FLAG epitope at its C terminus.
- 20
25 In HCC cells expressing this protein, insulin- or IGF-1-mediated TP occurs on the mutant protein, while TP of endogenous hIRS-1, as well as of other substrates for insulin- or IGF-1-induced TP (e.g., Shc), is substantially decreased. Insulin- or IGF-1-mediated activation of other signal transducers such as MAPK and PI3K is also diminished. The HCC cells expressing this inhibitory protein display a loss of their tumorigenic phenotype. Additionally, while HCC cells form tumors in nude mice,
- 30

the HCC cells expressing this protein do not. This Example demonstrates that dominant negative mutants of IRS-1 will be useful in the study and treatment of HCC.

As is shown in Fig. 1 and the Example described below, the amino-terminal and carboxy-terminal regions of IRS-1 perform different functions in the native molecule. The dominant negative mutants of the invention retain the functional properties of the amino-terminus, while shedding those of the carboxy-terminus. The amino-terminus, up to about amino acid residue 516 in hIRS-1, is the site of two activities which are important to retain in the mutant analogs of the invention: (i) it contains tyrosine residues which are tyrosine phosphorylated, at the expense of the tyrosine phosphorylation of the native molecule; and (ii) it contains the pleckstrin homology domain, which permits the mutant protein to be brought into close enough proximity to cell membranes to inhibit the tyrosine phosphorylation of endogenous hIRS-1. The carboxy-terminal SH₂-binding region encompasses functions which are abolished in the dominant negative mutants of the invention. These functions are (i) binding to the SH₂ domains in target molecules; and (ii) activating those molecules via such binding. The SH₂-binding region contains SH₂-binding motifs ⁸⁹⁷YVNI (SEQ ID NO:2), ¹¹⁸⁰YIDL (SEQ ID NO:3), ⁶¹³YMPM (SEQ ID NO:4), and ⁹⁴²YMKM (SEQ ID NO:5), which bind respectively to Syp, Grb2, PI3K, and NCK. Preferably, in the dominant negative inhibitors of the invention, these motifs either have been deleted, or have been rendered unable to bind their respective receptors by amino acid substitutions that change the motifs' conformation.

In order to retain sufficient amino-terminal function for phosphorylation inhibition activity, the mutants of the invention should contain at least 315,

more preferably 460, and even more preferably 516 amino acid residues of the 516 amino acid-long amino-terminus of hIRS-1. In order to abolish the SH₂-binding motifs, amino acids 517-1243 of the carboxy-terminal region of
5 the molecule can be deleted or rendered inactive by critical amino acid substitutions or deletions.

Amino acid substitutions or deletions are permissible in the amino-terminal region as well, provided that the substitutions or deletions do not
10 interfere with the enzymatically-driven phosphorylation reaction, or with the bringing of the mutant molecules into close proximity with cell membranes. The FLAG epitope can be eliminated or replaced with another epitope similarly designed to facilitate identification
15 or purification of the mutant molecules.

Any mammalian cells that has an overactive IRS-1 signal transduction pathway (e.g., a signal transduction pathway activated by insulin, IGF-1, interferon, or growth hormone) can be treated with a mutant of the
20 invention. Exemplary cells are tumor cells of the gastrointestinal system (e.g., tumor cells in esophagus, stomach, pancreas, gall bladder, liver, and colon), lung cancer cells, and brain tumor cells. Targeting of the inhibitory proteins to the cells may be achieved by local
25 injection (e.g., into the hepatic portal vein for treatment of liver) of liposomes or other carriers (e.g., microspheres) that contain the inhibitory proteins. For enhanced targeting, the liposomes may be coated with molecules which function as ligands of tissue-specific
30 receptors. An example of such a receptor is the hepatic asialoglycoprotein receptor, useful ligands of which include asialo-orosomucoid and (poly)L-lysine-asialo-orosomucoid (Spiess, *Biochemistry* 29(43):10009-10018, 1990; Wu et al., *J. Biol. Chem.* 267(18):12436-12439,
35 1992; Wu et al., *Biotherapy* 3:87-95, 1991).

Alternatively, the protein may be introduced into a target cell by overexpressing within the cell a nucleic acid construct comprising a promoter sequence operably linked to a sequence encoding the protein. In this
5 method, the nucleic acid construct is derived from a non-replicating linear or circular DNA or RNA vector, or from an autonomously replicating plasmid or viral vector; or the construct is integrated into the host genome.

Any vector that can transfect a hepatocyte may be
10 used in the methods of the invention. Preferred vectors are viral vectors, including those derived from replication-defective hepatitis viruses (e.g., HBV and HCV), retroviruses (see, e.g., WO89/07136; Rosenberg et al., *N. Eng. J. Med.* 323(9):570-578, 1990), adenovirus
15 (see, e.g., Morsey et al., *J. Cell. Biochem., Supp.* 17E, 1993), adeno-associated virus (Kotin et al., *Proc. Natl. Acad. Sci. USA* 87:2211-2215, 1990), replication defective herpes simplex viruses (HSV; Lu et al., Abstract, page 66, *Abstracts of the Meeting on Gene Therapy*, Sept. 22-
20 26, 1992, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), and any modified versions of these vectors. Retroviruses are particularly useful since they typically target proliferating and not quiescent cells. Non-viral vectors, e.g., plasmid vectors, can also be
25 used. In one such system, the plasmid forms a molecular conjugate with poly-L-lysine by electrostatic forces. Poly-L-lysine covalently bonds to a ligand that binds to a receptor on tumor cells (Cristiano et al., *J. Mol. Med.* 73:479-486, 1995).

30 Methods for constructing expression vectors are well known in the art (see, e.g., *Molecular Cloning: A Laboratory Manual*, Sambrook et al., eds., Cold Spring Harbor Laboratory, 2nd Edition, Cold Spring Harbor, New York, 1989; *Current Protocols in Molecular Biology*,
35 Ausubel et al., eds., John Wiley & Sons, Inc., 1995).

In these vectors, promoters are operably linked to the nucleic acid sequence encoding an inhibitory protein of the invention. Any promoter that can direct a high level of transcription initiation in the target cells may be used in the invention. Non-tissue specific promoters, such as the cytomegalovirus (DeBernardi et al., *Proc. Natl. Acad. Sci. USA* 88:9257-9261, 1991, and references therein), mouse metallothionein I (Hammer, et al., *J. Mol. Appl. Gen.* 1:273-288, 1982), HSV thymidine kinase (McKnight, *Cell* 31:355-365, 1982), β -actin, and SV40 early (Benoist et al., *Nature* 290:304-310, 1981) promoters may be used in the invention. However, preferred in the invention are tissue-specific (e.g., hepatocyte-specific) promoters, the use of which ensures that the proteins are expressed primarily in the target tissue (e.g., liver). Exemplary hepatocyte-specific promoters are the albumin, α -fetoprotein, alpha-1-antitrypsin, retinol-binding protein, and asialoglycoprotein receptor promoters. Viral promoters and enhancers that include those derived from herpes simplex viruses (types I and II), hepatitis viruses (A, B, and C), and Rous sarcoma virus (RSV; Fang et al., *Hepatology* 10:781-787, 1989) may also be used in the invention.

The above-described nucleic acid constructs and vectors can be introduced into target cells as naked DNA, or by liposome fusion or erythrocyte ghosts. Alternatively, the nucleic acid constructs can be coupled to ligands of tissue-specific receptors, and thereby enter the target cells via receptor-mediated endocytosis. For example, one could use a ligand which binds the hepatic asialoglycoprotein receptor, such as asialo-orosomucoid or (poly)L-lysine-asialo-orosomucoid. Alternatively, one can employ a viral-based vector as a means for introducing the nucleic acid into hepatocytes.

The inhibitory proteins of the invention can be produced in commercially significant amounts by recombinant methods employing cultured cells. The cells can be prokaryotes (e.g., *E. coli*) or eukaryotes (e.g., yeast, insect cells, or mammalian cells). The nucleic acid molecules encoding the inhibitory proteins and having appropriate expression control sequences can be introduced into the cultured cells by viral infection, receptor-mediated endocytosis, liposome fusion, biolistic transfer, electroporation, calcium phosphate precipitation, DEAE-Dextran transfection, or any other standard transfection technique. Extraction and purification of recombinant proteins produced by the transfected cells can be performed with techniques well known in the art, including, for example, immunoaffinity purification.

Therapeutic compositions containing the inhibitory proteins or nucleic acid molecules encoding these proteins can be administered to a patient with hepatocarcinoma, or prophylactically to a patient who, e.g., is infected with hepatitis B or C virus but has not yet shown symptoms of hepatocarcinoma. The therapeutic compositions of the invention may be used alone or in a mixture, or in chemical combination, with one or more materials, including cancer chemotherapeutics, other proteins or recombinant vectors that increase the biological stability of the proteins or the recombinant vectors, or agents that increase the therapeutic compositions' ability to penetrate hepatocytes selectively. The therapeutic compositions of the invention may be administered in a pharmaceutically acceptable carrier (e.g., physiological saline), which is selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers, as well as

pharmaceutical necessities for use in pharmaceutical formulations, are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field, and in the USP/NF.

5 The therapeutic compositions of the invention can be administered in dosages determined to be appropriate by one skilled in the art. It is expected that the dosages will vary, depending upon the pharmacokinetic and pharmacodynamic characteristics of the particular agent,
10 and its mode and route of administration, as well as the age, weight, and health (including renal and hepatic function) of the recipient; the nature and extent of the disease; the frequency and duration of the treatment; the type of, if any, concurrent therapy; and the desired
15 effect. It is expected that a useful dosage contains between about 0.1 to 100 mg of active ingredient per kilogram of body weight. Ordinarily, 0.5 to 50 mg, and preferably 1 to 10 mg of active ingredient (nucleic acid or protein) per kilogram of body weight per day, given in
20 divided doses or in sustained release form, is appropriate. If the protein or nucleic acid is delivered locally to the site of the tumor, a smaller dose can be used.

 The therapeutic compositions of the invention may
25 be administered to a patient by any appropriate mode, e.g., parenterally, intraperitoneally, or intravenously, as determined by one skilled in the art. Alternatively, it may be desired to administer the treatment surgically to the target tissue, e.g., by implantation of a
30 biodegradable capsule. The treatments of the invention may be repeated as needed, as determined by one skilled in the art.

 The mutants of the invention can also be used in studies of the insulin/IGF-1 signal transduction pathway
35 and its relationship with other signal transduction

pathways in mammalian cells. For instance, a mutant of the invention can be used to block the insulin-activated signal transduction pathway in a cell, so that alternative signal transduction pathways in the cell can be studied. Methodology used for such studies is well known in the art of signal transduction.

The following example is meant to illustrate the methods and materials of the present invention. Suitable modifications and adaptations of the described conditions and parameters which are obvious to those skilled in the art are within the spirit and scope of the present invention.

EXAMPLE

Materials and Methods

15 Reagents and Cells

FOCUS is a poorly differentiated human cell line derived from a HBV (hepatitis B virus) positive hepatocellular carcinoma tumor (Nishiyama et al., *Biochem. Biophys. Res. Commun.* 183:280-285, 1992). The FOCUS cells were employed as a source of mRNA for a cDNA library used to clone the hIRS-1 cDNA. HepG2 (hepatoblastoma) cells, which are well differentiated, were cultured in Dulbecco's modified Eagle's media (DMEM; Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma), and HuH-7 HCC cells, which are moderately differentiated, were cultured in RPMI medium 1640 (Mediatech) supplemented with 30 nM Na_2SeO_3 (serum free) or with 10% FBS. For immunoprecipitation and immunoblot studies, anti-IRS-1 rabbit polyclonal antibody (Upstate Biotechnology), anti-phosphotyrosine antibody conjugated to horseradish peroxidase (PY20H; Transduction Laboratories), anti-Shc rabbit polyclonal antibody (Transduction Laboratories), anti-insulin receptor rabbit polyclonal antibody (provided by Dr. X. F. Zhang),

anti-FLAG M2 mouse monoclonal antibody (Kodak), anti-Grb2 rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-Syp/PTP2C mouse monoclonal antibody (Transduction Laboratories) and anti-p85 PI3K rabbit polyclonal antibody (Upstate Biotechnology Inc.) were employed. Mitogen-activated protein kinase (MAPK) activity was measured using myelin basic protein (MBP; Sigma) as a specific substrate.

Construction of hIRS-1 Mutants

DNAs encoding various hIRS-1 deletional mutants were sequentially constructed using PCR or restriction enzyme digestion. A FLAG epitope (DYKDDDDK; SEQ ID NO:6) was added to the C-terminus by PCR. Each cDNA was subcloned into the pCDNA3 plasmid (Invitrogen) between the CMV promoter and the SV40 poly A region. The nucleotide sequences of the DNAs were confirmed by sequencing with Sequenase 2.0 (United States Biochemical Inc.).

Cell Culture and Transfection Studies

Parental FOCUS cells, HepG2 cells, and HCC transfectants (He et al., *In Vitro* 20:493-504, 1984) were cultured in DMEM supplemented with 10% heat-inactivated FBS. HCC cells (1×10^6) grown in 3.5 cm petri dishes were transfected with 2 μ g of one of the various hIRS-1 mutant constructs or the pcDNA3 vector alone (mock) using Lipofectamine (Life Technologies, Inc.) according to manufacturer's instructions. Two days after transient transfection, cells were serum starved for 12 hr, and then treated with 100 nM insulin. To select for stable transfectants, transfected cells were split 1:10 and grown in the presence of 800 μ g/ml G418. Resistant colonies were isolated.

Immunoblot and Immunoprecipitation Analysis

HCC transfectants were serum starved for 24 hr, and then treated with insulin, IGF-1 or EGF-1. The cells

were subsequently lysed in a Triton-lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton, 2 mM EGTA, 10 mM EDTA, 100 mM NaF, 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM NaVO_4 , 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml aprotinin, 5 3.5 mg/ml pepstatin A, and 25 mg/ml leupeptin. Cell lysates containing about 100 μg of total protein were electrophoresed on SDS-polyacrylamide gel, and analyzed by Western blot analysis using Immobilon-P membrane (Millipore Corp.). For immunoprecipitation studies, cell 10 lysates containing about 500 μg of protein were incubated with specific antibodies and precipitated with protein A-agarose beads. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and analyte proteins were detected with specific antibodies.

15 Mitogenic and Anchorage-Independent Growth Assays

HCC cells transfected with the various IRS-1 constructs were plated at a concentration of 1×10^5 cells in 24-well plates. Twenty-four hours later, the medium was replaced with DMEM containing 0.25% FBS. Cells were 20 incubated with or without 100 nM insulin or 100 ng/ml IGF-1 for 18 hr, and pulse-labeled with 1 mCi/ml [^3H]-thymidine in DMEM for 3 hr. The labeled cells were washed, and re-suspended in 1 ml of PBS containing 0.1% SDS and 10% trichloroacetic acid (TCA). The TCA- 25 insoluble cell pellet was solubilized in 1 N NaOH and neutralized with HCl. The amount of [^3H]-thymidine incorporated into DNA was determined by liquid scintillation counting.

The ability of transfected cells to exhibit 30 anchorage-independent growth was determined by plating 1×10^3 cells in DMEM containing 10% FBS and 0.4% soft agar (FMC Bioproducts). The DMEM/FBS/agar medium was overlaid on a bottom agar medium containing DMEM, 10% FBS and 0.53% agar. Anchorage-independent growth of the parental 35 and clonal stable transfectants was assessed by counting

the number of colonies formed in soft agar 2 weeks after seeding.

Tumor Formation in Nude Mice

To investigate tumorigenicity of the HCC
5 transfectants, 1×10^7 cells of the parental (i.e.,
transfected with mock plasmid DNA) and Δ ChIRS-1 FOCUS HCC
cell clones in 0.1 ml PBS were injected subcutaneously
into the backs of nude mice. The mice were observed for
4 weeks, and solid tumor formation was determined.

10 MAPK and PI3K Assays

Insulin or IGF-1-induced MAPK and PI3K activation
was measured using cell lysates derived from transfected
cells that had been incubated with or without 100 nM
insulin or 100 ng/ml IGF-1 for 10 min after 24 hr of
15 serum starvation. MAPK enzymatic activity was measured
as previously described (Baltensperger et al., *Science*
260:1950-1952, 1993). Briefly, lysates of cells with or
without hormone stimulation were electrophoresed on
SDS-polyacrylamide gel containing 0.5 mg/ml MBP. SDS was
20 subsequently removed from the gel by washing the gel with
50 mM Tris-HCl (pH 8.0) containing 20% 2-propanol for 1
hr and then with 50 mM Tris-HCl (pH 8.0) containing 5 mM
2-mercaptoethanol for an additional hour at 20°C. MAPK
in the gel was denatured by treatment with 6M guanidine
25 HCl and 50 mM Tris-HCl (pH 8.0) for 1 hr at 20°C, and
renatured in 50 mM Tris-HCl (pH 8.0) containing 0.04%
Tween-40 and 5 mM 2-mercaptoethanol. The gel was then
incubated in a kinase buffer [40 mM HEPES (pH 8.0), 2 mM
dithiothreitol, 0.1 mM EGTA, 20 mM MgCl_2] for 1 hr at
30 25°C. Phosphorylation of MBP was performed by incubating
the gel with a kinase buffer containing 25 μCi of [γ - ^{32}P]
ATP (Dupont) for 1 hr at 25°C. The gel was washed in 5%
(w/v) TCA solution containing 1% sodium pyrophosphate.
MAPK-induced phosphorylation of MBP was determined by
35 autoradiography.

To measure PI3K activity, 500 μ g of cell lysate was immunoprecipitated with polyclonal antibody against the p85 subunit of PI3K, and then incubated with protein A-SEPHAROSE beads. The immunoprecipitates were washed
5 twice with cell lysis buffer, once with 1% NP40-PBS, once with 100 mM Tris-HCl (pH 7.4) containing 0.5 M LiCl, and twice with 10 mM Tris-HCl containing 100 mM NaCl. Samples were preincubated with a phosphoinositol (PI) solution (sonicated PI, 50 mM HEPES, 1 mM EGTA, 1 mM sodium
10 phosphate) and then reacted with 1 μ Ci of [γ - 32 P] ATP, 50 mM ATP and 10 mM $MgCl_2$ for 10 minutes. After termination of the reaction with HCl and $CHCl_3$ /methanol mixture, lipid phosphorylation was analyzed by thin-layer chromatography on silica gel plates coated with 1%
15 potassium oxalate. Radioactive signals on the gel plates were visualized by autoradiography.

Results

The N-terminus of hIRS-1 is Required for Its Tyrosyl Phosphorylation in HCC Cells

20 To determine the regions of hIRS-1 required for its TP in FOCUS cells, DNA constructs expressing N--terminal (designated hIRS-1 Δ 3-516 or Δ N-hIRS-1) and C-terminal (designated hIRS-1 Δ 517-1243 or Δ C-hIRS-1) truncated mutants of hIRS-1, each of which contained a
25 FLAG epitope at its C-terminus, were made (Fig. 1) and transiently transfected into FOCUS cells. Both hIRS-1 mutants have numerous potential TP sites in their sequences (Myers et al., *Trends Biochem. Sci.* 19:289-293, 1994; Sun et al., *Nature* 377:173-177, 1995). Western
30 blot analysis demonstrated that, following transfection of hIRS-1 Δ 3-516 and hIRS-1 Δ 517-1243 constructs into HCC cells, the mutant proteins were expressed with the predicted molecular size (approximately 100 kD and 60 kD, respectively) as determined by a C-terminal specific
35 antibody and anti-FLAG M2 antibody. Western blot analysis using an anti-phosphotyrosine antibody (aPY)

revealed that insulin stimulation caused TP in hIRS-1Δ517-1243 but not in hIRS-1Δ3-516. TP of hIRS-1Δ517-1234 was highly prominent in FOCUS transfectants. TP of hIRS-1Δ517-1243 was also apparent in another transiently transfected HCC cell line, HepG2. These results show that at least a portion of the amino-terminal 516 residues of hIRS-1 is required for insulin-stimulated TP of hIRS-1 in both FOCUS and HepG2 HCC cell lines.

10 TP of ΔC-hIRS-1 Requires the First PTB Domain

The N-terminus of hIRS-1 is composed of three functional domains: a pleckstrin homology (PH) domain and two PTB domains. To address the role of these domains in TP, constructs encoding hIRS-1 mutants with sequential truncations of these domains (Fig. 1) were prepared. The FOCUS cells were stably transfected with each mutant construct and 2 to 4 neomycin-resistant transfectant clones were established for each construct. Each mutant protein has potential TP sites in its sequence (Myers et al., *Trends Biochem. Sci.* 19:289-293, 1994; Sun et al., *Nature* 377:173-177, 1995). Western blot analysis using anti-FLAG M2 antibody confirmed that progressive truncation of these domains reduced the size of the expressed protein. Western blot analysis using aPY showed that TP of ΔC-hIRS-1, ΔC-hIRS-1Δ9-117, ΔC-hIRS-1Δ163-256, and ΔC-hIRS-1Δ9-117Δ320-457 mutant proteins was evident in insulin-stimulated FOCUS cells, whereas ΔC-hIRS-1Δ163-256, ΔC-hIRS-1Δ119-457, and ΔC-hIRS-1Δ4-296 mutant proteins were not tyrosyl phosphorylated to any appreciable extent. There was no change in the level of expression of the insulin receptor or endogenous hIRS-1 protein in all stable transfected and cloned cell lines. These studies demonstrate that the most amino-terminal PTB domain is required for TP of ΔC-hIRS-1 in FOCUS HCC cells.

Δ C-hIRS-1 inhibits insulin- and IGF-1-induced TP of endogenous hIRS-1

Subsequent to insulin (100 nM) stimulation for 5 minutes, the 180-190 kDa endogenous hIRS-1 protein was tyrosyl-phosphorylated in FOCUS cells transfected with mock plasmid DNA. However, in FOCUS cells stably transfected with Δ C-hIRS-1, the TP of endogenous hIRS-1 (immunoprecipitated by anti-hIRS-1 antibody) was substantially decreased, while the expressed Δ C-hIRS-1 mutant protein (immunoprecipitated by M2 antibody) was highly tyrosyl-phosphorylated. Endogenous hIRS-1 was rapidly dephosphorylated within 2 hr in insulin-deprived FOCUS cells transfected with mock plasmid DNA. The tyrosyl-phosphorylated Δ C-hIRS-1 protein was not dephosphorylated as rapidly as endogenous hIRS-1, and high levels of phosphorylation were observed after 8 hr of insulin deprivation. The dominant negative effect of Δ C-hIRS-1 on TP of endogenous hIRS-1 was not observed with other hIRS-1 mutant proteins (e.g., Δ C-hIRS-1 Δ 9-117, Δ C-hIRS-1 Δ 163-256, Δ C-hIRS-1 Δ 320-457, Δ C-hIRS-1 Δ 119-457, Δ C-hIRS-1 Δ 9-117 Δ 320-457, and Δ C-hIRS-1 Δ 4-296).

Notably, the Δ C-hIRS-1 protein co-immunoprecipitated with the β subunit of the insulin receptor, suggesting that the mutant molecule binds and occupies the insulin receptor. In this experiment, cells from two different clones of the Δ C-hIRS-1 transfectant were treated with or without insulin, and cell lysates of these cells were immunoprecipitated with an anti-insulin receptor antibody. The immunoprecipitates were immunoblotted with an anti-FLAG antibody (aIR/aFLAG) to detect the Δ C-hIRS-1 mutant protein in the immunoprecipitate. Other tyrosyl-phosphorylated mutant proteins were found not to co-immunoprecipitate with the β subunit of the insulin receptor.

Insulin and IGF-1 also stimulated TP of Shc in FOCUS cells transfected with mock plasmid DNA. Stable

FOCUS transfectants of Δ C-hIRS-1 (i.e., cell lines c1 and c2) showed a striking suppression of insulin- and IGF-1-induced TP of Shc. However, expression of Δ C-hIRS-1 did not affect TP of Shc induced by EGF stimulation. The result indicates that Δ C-hIRS-1 overexpression in HCC cells specifically inhibits insulin- and IGF-1-induced TP of endogenous cellular substrates in addition to hIRS-1.

Tyrosyl phosphorylation of endogenous hIRS-1 induced by insulin or IGF-1 leads to the activation of downstream molecules (such as MAPK and PI3K) which are involved in the signal transduction cascade. However, in FOCUS cells stably transfected with Δ C-hIRS-1, insulin-induced activation of MAPK and PI3K was diminished to levels found in non-insulin stimulated cells.

[3 H]-thymidine incorporation into DNA was measured in stable FOCUS transfectants in order to assess the effects of the various hIRS-1 mutants on insulin- and IGF-stimulated DNA synthesis. [3 H]-thymidine incorporation in response to insulin/IGF-1 treatment was increased approximately threefold in cells transfected with mock plasmid DNA. However, in each of the two Δ C-hIRS-1-transfected clones studied (i.e., c1 and c2), [3 H]-thymidine incorporation following insulin or IGF-1 treatment remained at a level similar to that of cells not treated with the hormone (i.e., quiescent cells). [3 H]-thymidine incorporation was not substantially inhibited in FOCUS cell lines stably transfected with the other mutant constructs.

Δ C-hIRS-1 expression changes the characteristics of the malignant phenotype

Parental FOCUS cells exhibit characteristics of the malignant phenotype including anchorage independent cell growth (Barrett et al., *Proc. Natl. Acad. Sci. USA* 75:3761-3765, 1978; He et al., *In Vitro* 20:493-504, 1984). As shown in Table 1, FOCUS cells transfected with mock plasmid DNA formed colonies in soft agar. However,

individual clones derived from ΔC-hIRS-1 transfected FOCUS cells demonstrated very low efficiency of colony formation in soft agar, indicating the loss of the capacity for anchorage independent cell growth. Cloned
5 cell lines transfected with other mutant hIRS-1 constructs all retained to a significant degree the ability to grow in an anchorage-independent manner.

Parental FOCUS cells are poorly differentiated, and exhibit a large irregular flattened shape, multiple
10 mitotic figures, and pleomorphic nuclei with prominent nucleoli. FOCUS cells transfected with mock plasmid DNA retained these characteristics. Like parental cells, the mock transfected cells grew rapidly without contact inhibition and formed multi-layered structures when they
15 reached confluence. In contrast, stable transfection with ΔC-hIRS-1 substantially altered the morphological appearance of the FOCUS cells. The cloned cell lines c1 and c2 exhibited differentiated morphology such as sinusoid formation, and in addition exhibited contact
20 inhibition of cell growth. These cells, which were much smaller than parental cells and had a polygonal morphology with normal-appearing nuclei, had an appearance reminiscent of adult hepatocytes in primary cultures (Bissell et al., *J. Clin. Invest.* 79:801-812,
25 1987).

A similar alteration of phenotype was also observed in HuH-7 cells stably transfected with ΔC-hIRS-1. HuH-7 cells were derived from a human tumor not related to chronic HBV infection, and can be grown
30 under serum free conditions (Nakabayashi et al., *Cancer Res.* 42:3858-3863, 1982). Following treatment with 100 ng/ml IGF-1, expression and TP of ΔC-hIRS-1 were observed in cells of stably transfected HuH-7 cell lines h1 and h2. Expression of ΔC-hIRS-1 inhibited IGF-1-induced TP
35 of endogenous hIRS-1. HuH-7 cells transfected with mock

plasmid DNA had a pleomorphic and fibroblastoid appearance in a serum-free medium as well as in a medium containing 10% FBS. H1 and h2 cells, however, demonstrated a differentiated polygonal cellular morphology and formed flat sheets of cells, exhibiting contact inhibition with a sinusoidal growth pattern reminiscent of adult hepatocytes in culture.

Finally, the ability of parental and transfected FOCUS cells to form solid tumors was assessed in nude mice as previously described (He et al., *In Vitro* 20:493-504, 1984). 1×10^7 FOCUS cells transfected with mock plasmid DNA were injected subcutaneously into nude mice, and rapid tumor formation was observed at the injection site. The tumors reached a mean size of 2.5 cm within 28 days in 9 of 10 injected mice. In contrast, none of the mice injected with c1 or c2 cells developed tumors (Table 2).

Table 1: Anchorage-independent growth of FOCUS transfectants

5	Clone	Efficiency of colony formation in soft agar (%) *
	mock	7.1 ± 0.5
10	ΔC-hIRS-1 c1	0.2 ± 0.1
	ΔC-hIRS-1 c2	0.1 ± 0.1
	ΔC-hIRS-1Δ9-117 c1	4.3 ± 1.1
	ΔC-hIRS-1Δ9-117 c2	3.6 ± 0.7
	ΔC-hIRS-1Δ163-256 c1	6.8 ± 1.0
15	ΔC-hIRS-1Δ163-256 c2	7.2 ± 0.8
	ΔC-hIRS-1Δ320-457 c1	4.0 ± 0.6
	ΔC-hIRS-1Δ320-457 c2	3.1 ± 0.3
	ΔC-hIRS-1Δ119-457 c1	8.2 ± 1.1
	ΔC-hIRS-1Δ119-457 c2	7.6 ± 1.3
20	ΔC-hIRS-1Δ9-117Δ320-457 c1	5.1 ± 0.5
	ΔC-hIRS-1Δ9-117Δ320-457 c2	4.2 ± 0.4
	ΔC-hIRS-1Δ4-296 c1	6.6 ± 0.9
	ΔC-hIRS-1Δ4-296 c2	7.1 ± 0.8

25 *Efficiency of colony formation is represented by percentage of cells that formed colonies in each well. The data were obtained from duplicate experiments.

Table 2: Tumor formation by FOCUS transfectants

Clone	Tumors/mice*
5 mock plasmid	9/10
ΔC-hIRS- 1 c1	0/9
ΔC-hIRS- 1 c2	0/9

—
*Tumorigenicity was determined by subcutaneous injection
10 of 1×10^7 cells into nude mice and measurement of tumor
size at week 4 after injection.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is
5 intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A dominant negative mutant of a mammalian IRS-1.

2. The mutant of claim 1, wherein the mutant
5 comprises the pleckstrin homology domain and the two
phosphotyrosine binding domains of said IRS-1, and lacks
at least one functional SH₂-binding motif of said IRS-1.

3. The mutant of claim 2, wherein the mutant
does not bind a SH₂ domain of Syb, Grb2, PI3K, or NCK.

10 4. The mutant of claim 1, wherein the
mammalian IRS-1 is a human IRS-1.

5. The mutant of claim 2, wherein the
mammalian IRS-1 is a human IRS-1, and the mutant
comprises 460 amino acid residues from the amino-terminal
15 half of the human IRS-1, and lacks at least 300 amino
acid residues from the carboxy-terminal half of the human
IRS-1.

6. The mutant of claim 2, wherein the mutant
further comprises a heterologous sequence 4-25 amino acid
20 residues in length, said heterologous sequence comprising
an epitope to which an antibody binds.

7. The mutant of claim 5, wherein the amino
acid sequence of the mutant is SEQ ID NO:7.

8. The mutant of claim 5, wherein the amino
25 acid sequence of the mutant is SEQ ID NO:8.

9. A DNA molecule encoding the mutant of
claim 1.

10. A DNA molecule encoding the mutant of
claim 7.

5 11. A DNA molecule encoding the mutant of
claim 8.

12. A method of inhibiting tyrosyl
phosphorylation of an IRS-1 in a mammalian cell, the
method comprising introducing into the cell an amount of
10 a protein effective to inhibit tyrosyl phosphorylation of
said IRS-1, wherein the protein is a dominant negative
mutant of said IRS-1.

13. The method of claim 12, wherein the mutant
contains the pleckstrin homology domain and the two
15 phosphotyrosine binding domains of said IRS-1, and lacks
at least one functional SH₂-binding motif of said IRS-1.

14. The method of claim 13, wherein the mutant
does not bind a SH₂ domain of Syb, Grb2, PI3K, or NCK.

15. The method of claim 12, wherein the
20 mammalian cell is a human cell and the IRS-1 is a human
IRS-1.

16. The method of claim 15, wherein the
mammalian cell is a human hepatic cell.

17. The method of claim 15, wherein the mutant comprises 460 amino acid residues from the amino-terminal half of the human IRS-1, and lacks at least 300 amino acid residues from the carboxy-terminal half of the human
5 IRS-1.

18. The method of claim 17, wherein the amino acid sequence of the protein is SEQ ID NO:7.

19. The method of claim 17, wherein the amino acid sequence of the protein is SEQ ID NO:8.

10 20. The method of claim 12, wherein the protein is introduced into the cell by expression from a recombinant expression construct within the cell.

21. A method of reversing a malignant phenotype of a mammalian tumor cell, the method
15 comprising introducing into the cell an amount of a protein effective to reverse a tumorigenic phenotype of the cell, wherein the protein is a dominant negative mutant of an IRS-1 in the cell.

22. The method of claim 21, wherein the mutant
20 comprises the pleckstrin homology domain and the two phosphotyrosine binding domains of said IRS-1, and lacks at least one functional SH₂-binding motif of said IRS-1.

23. The method of claim 22, wherein the mutant does not bind a SH₂ domain of Syb, Grb2, PI3K, or NCK.

25 24. The method of claim 21, wherein the mammalian cell is a human cell and the IRS-1 is a human IRS-1.

25. The method of claim 24, wherein the mammalian cell is a human hepatic cell.

26. The method of claim 24, wherein the protein comprises 460 amino acid residues from the amino-
5 terminal half of the human IRS-1, and lacks at least 300 amino acid residues from the carboxy-terminal half of the human IRS-1.

27. The method of claim 26, wherein the sequence of the protein is SEQ ID NO:7.

10 28. The method of claim 26, wherein the sequence of the protein is SEQ ID NO:8.

29. The method of claim 21, wherein the protein is introduced into the cell by expression from a recombinant expression construct within the cell.

15 30. The method of claim 21, wherein the malignant phenotype is characterized by at least one of the following: growth without contact inhibition, anchorage-independent growth, increased rate of proliferation as compared to a corresponding normal cell,
20 and ability to form a tumor in a nude mouse.

31. A method of treating a malignancy in which tyrosyl phosphorylation of IRS-1 plays a causative role, said method comprising administering to a patient having such malignancy a therapeutic composition comprising a
25 dominant negative mutant of said IRS-1, or a DNA encoding such an mutant.

32. The method of claim 31, wherein said mutant is tyrosine phosphorylated in vivo, thus decreasing the tyrosine phosphorylation of said IRS-1.

33. The method of claim 31, wherein said
5 mutant does not bind to a SH₂ domain of Syp, Grb2, PI3K, or NCK.

34. A therapeutic composition comprising a dominant negative mutant of a human IRS-1, or a DNA encoding such an mutant, admixed with a pharmaceutically
10 acceptable carrier.

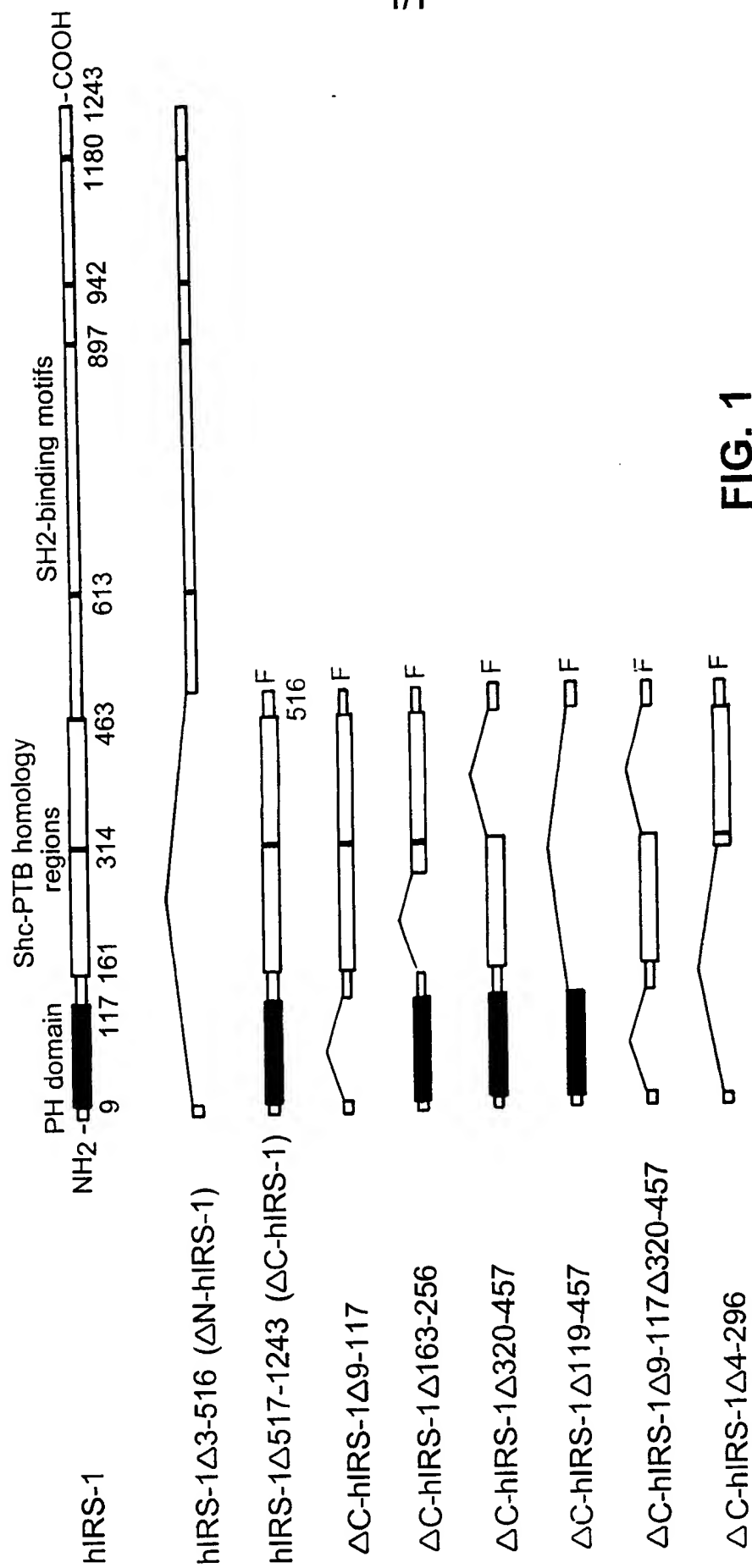


FIG. 1

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/00, 43/04; C07K 1/00, 5/00, 7/00, 14/00, 16/00, 17/00

US CL : 514/14, 44; 530/324, 350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/14, 44; 530/324, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAFT, C. R. Deletion of 343 Amino Acids from the Carboxyl Terminus of the β -Subunit of the Insulin Receptor Inhibits Insulin Signaling. Biochemistry. 1994, Vol. 33, No. 31, pages 9143-9151, especially page 9143.	1-34
A	SANCHEZ-MARGALET, V. Role of p85 Subunit of Phosphatidylinositol-3-Kinase as an Adaptor Molecule Linking the Insulin Receptor to Insulin Receptor Substrate 1. Molecular Endocrinology. 1995, Vol. 9, No. 4, pages 435-442, especially page 435.	1-34
A	YENUSH, L. Functional Domains of the Insulin Receptor Responsible for Chemotactic Signaling. The Journal of Biological Chemistry. 07 January 1994, Vol. 269, No. 1, pages 100-104, especially page 100.	1-34



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

17 FEBRUARY 1998

Date of mailing of the international search report

06 MAR 1998

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